【DESCRIPTION】

【Invention Title】

THE USAGE OF MADS-BOX GENES IN FRUIT & SEED DEVELOPMENT BY REGULATING ACTIVE GIBBERELLIN SYNTHESIS

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【Technical Field】

The present invention relates to a novel use of a MADS-box gene. In particular, it relates to the use of a MADS-box gene selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain, for the regulation of fruit and seed development.

【Background Art】

In the most of angiosperm plants, fruit provides a suitable environment for seed maturation and often a mechanism for the dispersal of mature seeds.

The Fruit development usually starts with development and fertilization of ovary, which occurs by a variety of chemical and physiological changes (Dong Y-H et al., J. Amer. Soc. Hort. Sci., 1997, 122:752-757). After

the completetion of fertilization, an egg cell develops into an embryo, polar nuclei develop into endosperm and an ovule develops into seed. An ovary develops into fruit by the differentiation and growth of a cell and

5   transformation and accumulation of reserve substances therein. However, there are some cases where not only an ovary but also other parts of a floral organ such as receptacle are involved in the fruit development. Such fruits are called pome fruits and an apple is an example.

10   In general, the development of ovary into fruit is also accompanied by a seed formation, however, in the case of fruits without seed, seed does not form even after the fertilization and only the ovary itself develops. The forming a fruit without seed is called parthenocarpy.

15   Parthenocarpy occurs when ovary develops into a fruit in the absence of pollination (citrus, banana, pineapple, etc) or fertilization of an egg cell after pollination (some orchidaceae), or an embryo is aborted after the fertilization (grapes, peach, cherry etc).

20   MADS-box genes represents a gene family composing 30 or more amino acid sequences encoding a transcription factor containing a conserved region called MADS-domain. Many of those genes have been shown to be involved in the differentiation of a flower and other various organs in a

25   plant by regulating transcription. Specifically, MADS-box

genes are classified into group A, group B and group C, according to their respective functions (ABC model). Genes of group A take responsible for the development of sepal and petal, genes of group B for the development of petal and stamen, and genes of group C for the development of stamen and pistil. Among MADS-box genes derived from *Arabidopsis thaliana*, *APETALA1* (AP1) belongs to group A, *APETALA* (AP3) and *PISTILL* (PI) have been confirmed to belong to group B, and *AGAMOUS* (AG) is classified into group C (Gunter Theissen *et al.*, *Plant Molecular Biology*, 2000, 42: 115-149). Further, *Floral binding protein* 7 (FBP7) and *floral binding protein 11* (FBP11) which are found to be involved in the development of ovule have been classified into new group D (Gerco C. Angenent *et al.*, *Plant Cell*, 1995, 7: 1569-1582).

Gibberellin is a class of diterpenoid plant hormone, designated as GA1 to GAn depending on its kind. The basic structure of Gibberellin consists of 4 rings and contains 20 carbon atoms. Gibberellin plays an essential role in the development and growth of a plant, particulary, in the development of seed, stimulation of germination, growth of a stem, generation and growth of a flower, anthocyanin biosynthesis, fruit-setting, etc (Richard Hooley, *Plant Mol. Biol.*, 1994, 26: 1529-1555). The growth promotion by

gibberellin is not by stimulating the cell division but by stimulating an elongation and enlargement of a cell. In addition, gibberellin stimulates the formation of flower bud or anthesis. And, it is also used for vernalization

5   and long day treatment (Peter Hedden *et al.*, *Annu.Rev.Physiol.Plant Mol. Biol.*, 1997, 48:431-460).

Such functional activities of gibberellin, for example, in the growth of a plant, germination of a dormant seed, promotion of the growth of a dormant bud and

10 parthenocarp in a plant as mentioned above have been widely utilized in farms to increase productivity by accelerating the growth of an herbaceous plant or to increase the size of a fruit.

During the biosynthesis of Giberellin,

15 monooxygenases synthesize GA_{12} and GA_{53} in the early stage of the giberrelin synthesis, which are subseqently converted into C19-GA having physiological activities by GA 20-oxidase and 3β-hydroxylase resulting in the production of biologically active giberreline.

20 Specifically, GA 20-oxidase oxidizes 20th carbons of GA_{12} and GA_{53} step by step, providing the backbone structure of C-19 in gibberellin, and 3β-hydroxylase catalizes 3β-hydroxylation reaction on the C-19 structure of gibberellin thus obtained by GA 20-oxidase, producing

biologically active gibberellin (Neil Olszewski *et al.*, *Plant Cell*, 2002, 14: S61-S80).

The expression of GA 20-oxidase involved in biosynthesis of active gibberellin is negatively feedback regulated by active gibberellin. Precisely, the level of expression of mRNA transcripts for GA 20-oxidase is decreased as the active gibberellin synthesis is increased. On the contrary, the level of expression of mRNA transcripts for GA 20-oxidase is increased as the active gibberellin synthesis is decreased, resulting in the precise regulation of the amount of GA 20-oxidase synthesized (Martin *et al.*, *Planta*, 1996, 200(2): 159-66; Neil Olszewski *et al.*, *Plant Cell*, 2002, 14: S61-S80; Victor B. *et al.*, *Plant physiol.*, 2003, 132: 1283-1291). For example, *Le20ox-1*, a GA 20-oxidase isolated from tomato, converts C-20 gibberellin into C-19 gibberellin that is subsequently activated by 3β-hydroxylase. When the active gibberellin is over-synthesized, the expression of *Le20ox-1* mRNA is decreased, by the negative feedback regulation, to reduce the amount of *Le20ox-1* enzyme synthesized. Thus, C-19 gibberellin synthesis is reduced with the decrease of the amount of *Le20ox-1* enzyme synthesized, resulting in the regulation of the amount of active gibberellin (Mariken Rebers *et al.*, *Plant J.*, 1999,

17(3): 241-250). So, *low level expression of Le20os-1* mRNA indicates that active gibberellin is over-synthesized.

It has been reported that *RIN* gene plays a key role in ethylene synthesis during ripening of tomato fruit. Thus, the decrease of the expression of *RIN* gene means the decrease of ethylene synthesis during ripening of the fruit. That is, the decrease of ethylene synthesis by the introduced gene results in the delay of ripening (R.C. Herner *et al.*, *Plant Physiol.*, 1973, 52: 38-42; Julia Vrebalov *et al.*, *SCIENCE*, 2002, 296: 343-346).

At present, the effect of MADS-box genes on the development of fruit and seed has not been fully studied. Although the detailed mechanism of fruit development has yet to be found, it has been shown that several MADS-box genes are expressed in fruits and seeds. It has been recently reported that *AGAMOUS-like 15* (AGL15) gene directly controls the expression of gene involved in gibberellin synthesis during seed germination of *Arabidopsis thaliana* (Huai Wang *et al.*, *Plant Cell*, 2004, 16: 1206-1219).

Up to now, a number of researchers have developed various transgenic plants by gene manipulation in plants in order to increase the productivity of crops or to

develop new cultivars. Further, many transgenic plants
that have been proved to be safe have been cultivated and
allowed on the market for people to consume. Genetic
operation in relation to fruits and seeds has the
5   industrial need, since it can increase the productivity of
fruit and seed and be applied to the development of a
fruit and a horticultural crop by parthenocarpy.

Thus, the present invention has been completed by
10   isolating novel genes that can regulates the development
of fruit and seed by inhibiting or promoting the
expression of genes which control the synthesis of active
gibberellin, and producing a transgenic plant introduced
with genes showing enhanced germination efficiency and
15   parthenocarpic fruit formation.

【Disclosure】
【Technical Problem】
It is an object of the present invention to provide
20   a novel use of MADS-box gene as a regulator of fruit and
seed development.
【Technical Solution】
In order to achieve the above object, the present
invention provides a gene that regulates the fruit and
25   seed development by promoting or inhibiting the expression

of genes that control the synthesis of active gibberellin. The gene is selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain.

The present invention also provides an expression vector containing the above gene.

The present invention further provides transgenic plant cells containing the above gene.

The present invention also provides a transgenic plant whose fruit and seed development is regulated, produced by regenerating the above transgenic plant cells by tissue culture technique.

The present invention also provides a method of preparation of the transgenic plant.

The present invention also provides a regulator for fruit and seed development containing the above gene or a vector harboring the above gene as an effective ingredient.

The present invention also provides a regulator for active gibberellin synthesis containing the above gene or

a vector harboring the above gene as an effective ingredient.

Hereinafter, the present invention is described in
5   detail.

The present invention provides a gene that can regulates fruit and seed development by promoting or inhibiting the expression of genes that control the synthesis of active gibberellin. The gene is selected from
10   the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene
15   encoding an amino acid sequence having at least 85% homology within the region other than MADS-domain.

The gene of the present invention that contains a nucleotide sequence set forth in SEQ. ID. No 1 is
20   designated 'MdMADS14' gene (Fig. 1), and the gene having a nucleotide sequence set forth in SEQ. ID. No 2 is designated 'MdMADS16' gene (Fig. 2). It is preferred that MdMADS16 gene has 90 ~ 99% sequence homology with MdMADS14 gene. Also, MdMADS14 amino acids have at least about 88%
25   sequence homology with MdMADS16 amino acids (Fig. 3).

It has been shown that *MdMADS14* gene of the present invention is expressed only in the flowers of an apple tree, but its functions are unknown yet (C.G. van der Linden *et al.*, *Journal of Experimental Botany*, 2002, 53:

5   1025-1036). Meanwhile, *MdMADS 16* gene of the invention is a novel nucleotide sequence having 90 ~ 99% overall homology with *MdMADS 14* gene (although there is a little difference in homology rate according to a homology comparison program). As shown in Fig. 1 and 2, both

10   *MdMADS16* and 14 genes contain an open reading frame (ORF) coding for 242 amino acids and MADS-domain is located in *N*-terminal region of each gene. Overall Homology between *MdMADS16* and 14 amino acid sequences is 88.4% while the homology within MADS-domain region is 98.3%, and homology

15   within the region other than MADS-domain is at least 85% (Fig. 3).

Although the gene coding an amino acid sequence having at least 85% homology within the region other than MADS-domain of *MdMADS14* or *MDMADS16* is not represented by

20   a specific sequence list herein, it is easily guessed by a skilled people in this field. *MdMADS16* gene was proved to have at least 85% homology within the region other than MADS-domain and have same functions with *MdMADS14* gene, indicating that a gene coding an amino acid sequence

25   having over 85% homology in the region other than MADS-

domain with the gene of the present invention has equal functions to the gene of the invention.

In the preferred embodiment of the present invention, *MdMADS14* or *MdMADS16* genes were transferred into a tomato to investigate the function of those genes. From such studies, it has been shown that transformants with over-expressed *MdMADS14* gene resulted in two different phenotypes; one was designated *MdMADS14 sense 1* and the other *MdMADS14 sense 2*. In *MdMADS14 sense 1*, ripening of a fruit was delayed but germination of a seed was promoted. Specifically, as shown in Fig. 8 and Fig. 9, ripening of a fruit in *MdMADS14 sense 1* was delayed, compared with that of wild type, while a seed was already germinated in a fruit, suggesting that germination of a seed was much promoted.

Those characteristics were inherited from T1 generation to T2 generation. As shown in Fig. 10, the seeds (T2 generation) of *MdMADS14 sense 1* were stored at 4 ℃ within desiccator for one year, and then germination rate was determined and it was found that germination was promoted in T2 generation of *MdMADS14 sense 1*, compared with that of wild type. In the case of *MdMADS14 sense 2*, parthenocarpic fruit was formed and sepal was developed into fruit flesh. Further, *anti-sense* transformants in

which the expression of *MdMADS14* gene was suppressed showed the common phenotype of not bearing fruit. The above results indicate that *MdMADS14* gene is involved in fruit and seed development.

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Transgenic tomato in which *MdMADS16* gene was over-expressed resulted in also two different phenotypes corresponding to those shown in transformants over-expressing *MdMADS14* gene, each of which was designated

10 *MdMADS16 sense 1* and *MdMADS16 sense 2*. As shown in Fig. 14 ripening of a fruit was delayed in *MdMADS16 sense 1*, while a seed was germinated inside a fruit, similar to *MdMADS14 sense 1* shown above. For *MdMADS16 sense 2*, as shown in Fig. 15, it showed the same phenotype with *MdMADS*

15 *14 sense 2* in which sepal was changed into fruit flesh. Further, as shown in Fig. 16, *MdMADS16 anti-sense* in which the expression of *MdMADS16* gene was suppressed did not develop a fruit after pollination, like the case of *MdMADS14 anti-sense*. The above results indicate that

20 neither *MdMADS14 anti-sense* nor *MdMADS16 anti-sense* develop a fruit.

The expressions of *RIN* gene that is required to promote ethylene synthesis during ripening were

25 investigated in *MdMADS14 senses* and *MdMADS16 senses* and

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were compared with that of wild type. As shown in Fig. 17, the expression level of *RIN* gene in each of *MdMADS14 sense 1* and *MdMADS16 sense 1*, both having the phenotype of delayed ripening, was decreased.

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Moreover, the expression level of a gene coding for *LeGA20ox-1* (tomato GA20-oxidase), which can serve as an indicator to indirectly estimate the amount of active giberreline synthesized, in *MdMADS14 senses* and *MdMADS16 senses* were compared with that of wild type. As shown in Fig. 18, the expression level of *LeGA20ox-1* gene was much decreased in *MdMADS14 senses* and in *MdMADS16 senses* than in wild type. Such results indicate that more active gibberellin was synthesized in *MdMADS14 senses* and in *MdMADS16 senses* than in wild type. On the contrary, the expression level of *LeGA20ox-1* gene was much increased in *MdMADS14 anti-sense* and in *MdMADS16 anti-sense* than in wild type, as shown in Fig. 19. Those results indicate that active gibberellin was synthesized much less in *MdMADS14 anti-sense* and in MdMADS16 *anti-sense* than in wild type.

From this study, it has been demonstrated that *MdMADS14* gene and *MdMADS16* gene are highly homologous to each other and are belong to the same gene family.

Further, through the analysis of gene functions using the transgenic plants, it has also been demonstrated that those genes were involved in fruit and seed development and have activities of promoting or inhibiting active

5   gibberellin synthesis. In conclusion, *MdMADS14* gene and *MdMADS16* gene derived from apple in the present invention are novel having the function of regulating fruit and seed development, which are unknown function in MADS-box genes.

10   The present invention also provides an expression vector containing *MdMADS14* gene or *MdMADS16* gene.

It is preferred that said gene to be inserted into an expression vector is selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ.

15   ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino acid sequence having at least 85% homology within the region other than

20   MADS-domain.

Any conventional expression vector for plant transformation may be used for the insertion of the gene of the present invention. For the present invention, pGA1530 vector for plant transformation was used for the

25   insertion of *MdMADS14* gene (Sung S-K. *et al*, *Plant*

Physiology, 1999, 120:969-978). The pGA1530 vector contains 35S promoter, T7 terminator, and *npt II* (*neomycin phosphotransferase*), as a marker gene, which provides kanamycin resistance (Stanton B. Gelvin *et al*, *Plant*

5   *molecular Biology Manual*, 1988, A3:1-19). For the insertion of *MdMADS16* gene, pCAMBIA 2301 vector for plant transformation was used. The pCAMBIA 2301 vector contains 35S promoter, *nos* (*nopaline synthase*) terminator, and *npt II* (*neomycin phosphotransferase*), as a marker gene,

10   that provides kanamycin resistance.

In the preferred embodiment of the present invention, expression vectors were constructed by inserting *MdMADS14* gene into pGA1530 forwardly or reversely. And the constructed vectors were designated 'pMdMADS14' and

15   'pMdMADS14-R'. Expression vectors were also constructed by inserting *MdMADS16* gene into pCAMBIA 2301 forwardly or reversely, which were designated 'pMdMADS16' and 'pMdMADS16-R'.

The present invention further provides transformed

20   plant cells containing the above gene.

The plant cells that can be used for the introduction of an expression vector of the present invention are not limited to specific forms as long as they can be regenerated into a plant. The cells include,

25   for example, cultured cell buoyant, protoplast, leaf

section and callus. An expression vector of the present invention may be introduced into plant cells by one of conventional methods such as polyethylene glycol method, electroporation, Agrobacterium mediating transduction, and

5   particle bombardment, etc. In the preferred embodiment of the present invention, the expression vector was transferred into plant cells by using Agrobacterium containing the gene of the present invention, and in particular, *Agrobacterium tumefaciens* LBA4404 (A. Hoekema

10   *et al.*, 1983, *Nature*, 303, 179-181) was used in this invention. Agrobacterium containing the gene of the present invention was prepared by transforming the cell with pMdMADS14 or pMdMADS16, expression vectors of the present invention. Each resulting agrobacterium was

15   designated 'Agrobacterium tumefaciens LBA4404/pMdMADS14' and 'Agrobacterium tumefaciens LBA4404/pMedMADS16', which were deposited with Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession Nos:

20   *Agrobacterium tumefaciens* LBA4404/pMdMADS14; KCTC 10588BP, *Agrobacterium tumefaciens* LBA4404/pMdMADS16; KCTC 10589BP).

The expression vectors of the present invention can be transferred to any kind of plants that can form a fruit

25   or a seed. Particularly, it is preferred that the plant

is selected from a group consisting of food crops such as rice, wheat, barley, corns, soybean, potato, red bean, oat, sorghum; vegetables such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage,

5   melon, pumpkin, spring onion, onion, carrot; industrial crops such as ginseng, *Acanthopanax senticosus*, tobacco, cotton, sesame, sugar cane, sugar beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum,

10   apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily, tulip; forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and landscape plants such as flowers and

15   shrubs. It is more preferred that the plant for the introduction of an expression vector of the present invention is tomato plant. In a specific embodiment of the invention, each Agrobacterium transformed with pMdMADS14 or pMdMADS16 was added and incubated with

20   cotyledon sections of minitomato to obtain a transformed tomato cotyledon (cells) containing the expression vector of the present invention.

The present invention also provides a transgenic plant, whose fruit and seed development was controlled,

produced by regenerating the above transgenic plant cells by tissue culture technique

Regeneration of the plant cells by tissue culture technique to obtain a transgenic plant of the present invention may be carried out by conventional method of producing transgenic plant. In a specific embodiment of the present invention, tomato cotyledons incubated with agrobacterium having the expression vector containing the gene of the present invention were sub-cultured in a regeneration medium (MS, IAA 1 µM, zeatin 10 µM, sucrose 3%, cefotaxime 350 mg/ℓ, kanamycin 50 mg/ℓ, agar 0.7%). Shoots produced from the cotyledon sections were than collected and it was further incubated in rooting medium (MS 1/2, IAA 1 µM, sucrose 3%, kanamycin 50 mg/ℓ, agar 0.7%), followed by the selection of a transformant. Untransformed shoots underwent necrosis accompanied by color change while transformed shoots were growing normally with roots in. Shoots with roots were transferred to soil and acclimation. Finally, transgenic tomatoes each with the phenotype characterized by that sepal was changed into fruit flesh and parthenocarpic fruit was formed, with the phenotype showing promoted seed germination and delayed ripening, and with the phenotype characterized by that fruit and seed development was inhibited because ovule was not developed, were prepared.

From the above results, it was confirmed that MADS-box genes of the present invention could be effectively used for the regulation of fruit and seed development and parthenocarpic fruit production in plants by promoting or inhibiting an expression of genes that control the active gibberellin synthesis.

All the plants forming a fruit or a seed can be a target plant of the present invention, which is preferably selected from a group consisting of food crops such as rice, wheat, barley, corns, soybean, potato, red bean, oat, sorghum; vegetables such as Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, spring onion, onion, carrot; industrial crops such as ginseng, *Acanthopanax senticosus*, tobacco, cotton, sesame, sugar cane, sugar beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum, apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily, tulip; forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and the landscape plants such as flowers and shrubs.

Further, the present invention encompasses the off-springs and clones of the transgenic plants of the present

invention as well as fruits, seeds, ears, tubers, tuberous roots, column, callus or protoplasts derived therefrom.

The present invention also provides a preparation method of a plant whose fruit and seed development are regulated, comprising the step of:

A method of preparing a transgenic plant whose fruit and seed development was regulated, comprising the steps of:

1) Constructting an expression vector comprising the gene according to Claim 1;

2) Transferring the vector constructed in Step 1) into Agrobacterium;

3) Co-culturing the transformed Agrobacterium of step 2) with plant tissue; and

4) Regenerating the transformed tissue into a mature transgenic plant.

In step 1) of the preparation method of the present invention, a MADS-box gene that regulates fruit and seed development is preferably selected from the group consisting of a gene having a nucleotide sequence set forth in SEQ. ID. No 1 containing a nucleotide sequence encoding MADS-domain, a gene having a nucleotide sequence set forth in SEQ. ID. No 2 containing a nucleotide sequence encoding MADS-domain and a gene encoding an amino

acid sequence having at least 85% homology within the
region other than MADS-domain. In step 1), any
conventional expression vectors for plant transformation
may be used for cloning a MADS-box gene. In a specific
embodiment, pGA1530 and pCAMBIA 2301 vectors were
preferably used for the insertion of MdMADS14 gene
MdMADS16, respectively. In step 2), It is preferred that
Agrobacterium which mediates transferring the gene of the
present invention into plant cells is Agrobacterium
tumefaciens LBA4404 (A. Hoekema et al., 1983, Nature, 303,
179-181). It is preferred that Agrobacterium having an
expression vector containing MdMADS14 gene or MdMADS16
gene is 'Agrobacterium tumefaciens LBA4404/pMdMADS14'
(Accession No: KCTC 10588BP) and 'Agrobacterium
tumefaciens LBA4404/pMdMADS16' (Accession No: KCTC
10589BP), respectively.

In step 3), all the plants forming a fruit or a seed
can be a target plant of the present invention, which is
preferably selected from a group consisting of food crops
such as rice, wheat, barley, corns, soybean, potato, red
bean, oat, sorghum; vegetables such as Chinese cabbage,
radish, red pepper, strawberry, tomato, watermelon,
cucumber, cabbage, melon, pumpkin, spring onion, onion;
carrot; industrial crops such as ginseng, Acanthopanax
senticosus, tobacco, cotton, sesame, sugar cane, sugar

beet, *Perilla japonica*, peanut, rape; fruits such as apple, pear, orange, jujube, peach, kiwifruit, grapes, tangerine, persimmon, plum, apricot, bananas; floricultural crops such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily, tulip; forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue, perennial ryegrass; fiber crops such as cotton plant; and landscape plants such as flowers and shrubs.

The present invention also provides a fruit and seed development regulator containing MADS-box gene(s) or an expression vector having the gene(s) as an effective ingredient.

The present inventors analyzed the function of said genes by examining the phenotypes of *MdMADS14 senses*, *MdMADS16 senses* and their *anti-senses* in which the genes were suppressed.

Result from the study showed the following phenotypes: delayed ripening but promoted seed germination (*senses 1*), change of sepal into fruit flesh and formation of parthenocarpic fruits (*senses 2*) and inhibited fruit and seed development (*anti-senses*).

Particularly, for *senses 1*, germination speed and rate were investigated with seeds (T2 generation) of *senses 1*. As shown in Fig. 9 and Fig. 10, seed

germination was promoted, compared with that of wild type. *For senses 2,* as shown in Fig. 11, sepal was developed into fruit flesh and parthenocarpic fruit without seed was formed unlike wild type. Plants of *anti-senses* did not develop fruits, showing that floral organ development in the early stage was not affect (Fig. 12(a) and (b)) but seed development was inhibited after pollination and so fruits were not formed (Fig. 12(c)).

From the above results, it was confirmed that *MdMADS14* gene, *MdMADS16* gene and expression vectors containing the genes could be effectively used for the regulation of fruit and seed development in plants

The present invention also provides an active gibberellin synthesis regulator containing MADS-box gene(s) or an expression vector(s) harboring the gene(s) as an effective ingredient.

Le20ox-1 is a GA 20-oxidase isolated from a tomato, whose mRNA synthesis is negatively feedback regulated by active gibberellin. *Le20ox-1* is an enzyme that catalzes the conversion of C-20 gibberellin into C-19 gibberellin, which subsequently activated by 3β-hydroxylase producing active giberellin. When active gibberellin is synthesized in excess, it negatively regulates the mRNA expression of *Le20ox-1* by negative feedback, resulting in the decrease

in the production of *Le20ox-1* enzyme. In turn, C-19 gibberellin synthesis is reduced with the decrease in the mRNA expression of *Le20ox-1*, resulting in the regulation of active gibberellin synthesis.

5   The present inventors compared the expressions of *Le20ox-1* gene in *MdMADS14 senses* and *MdMADS16 senses* in order to investigate the expresson levels of the *Le20ox-1* gene in transformed tomatos (Fig. 18 and Fig. 19).

The results show that the expression of *Le20ox-1*
10   gene was much decreased in *MdMADS14 senses* and in *MdMADS16 senses* than in wild type. On the contrary, the expression of *Le20ox-1* gene was increased in *MdMADS14 anti-senses* and *MdMADS16 anti-senses*. It cleary indicates that the decrease in the expression of *Le20ox-1* gene in *MdMADS14*
15   *senses* and *MdMADS16 senses*, compared with wild type, is caused by negative feedback by over-expressed active gibberellin, while the increase in the expression of *Le20ox-1* gene in *MdMADS14 anti-senses* and *MdMADS16 anti-senses* results in the decrease in the active gibberellin
20   synthesis. Therefore, it was confirmed that *MdMADS14* and *MdMADS16* genes of the present invention and a vector containing those genes could be effectively used for the control of active gibberellin synthesis.

25   【Description of Drawings】

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Fig. 1 is a schematic diagram showing the nucleotide sequence of *MdMADS14* gene containing a nucleotide sequence coding for MADS-doamin and K-domain as well as the amino acid sequence encoded thereby.

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Fig. 2 is a schematic diagram showing the nucleotide sequence of *MdMADS16* gene containing a nucleotide sequence coding for MADS-domain and K-domain as well as the amino acid sequence encoded thereby.

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Fig. 3 is a schematic diagram showing the homology between amino acid sequences of *MdMADS14* and *MdMADS16*.

Fig. 4 is a phylogenetic tree showing the relation
15   of *MdMADS14* and *MdMADS16* gene to other MADS-box genes in the same family. AP1: APETALA1 sub-family, SQUA: SQUAMOSA sub-family, AGL2: AGAMOUS-LIKE2 sub-family, AGL6: AGAMOUS-LIKE6 sub-family, AG: AGAMOUS sub-family.

20   Fig. 5 is a set of graphs showing the results from real-time PCR in which the mRNA level of *MdMADS14* and *MdMADS16* genes in each developmental stage of leaf, reproductive organ and fruit are quantified and compared.

A: Result of real-time PCR with cDNA from leaves of
25   an apple,

B: Result of real-time PCR with cDNA from flower bud of an immature apple,

C: Result of real-time PCR with cDNA from flower of a mature apple,

5   D: Result of real-time PCR with cDNA from fruit of an immature apple,

E: Result of real-time PCR with cDNA from fruit of a mature apple,

F: A graph showing the melting temperature of each

10   PCR product, obtained through melting curve analysis during real-time PCR,

G: A graph showing the expression profile of *MdMADS14* gene in each organ and in each developmental stage based on the results of graphs A - E,

15   H: A graph showing the expression profile of *MdMADS16* gene in each organ and in developmental stage based on the results of graphs A - E.

a - leaves;

b - flower buds with developing floral organ

20   primordia;

c - flower buds with developing anthers and fused carpels;

d - fruits (7 to 10 days after pollination);

e - ripe fruit.

25

Fig. 6 is a set of photographs showing the expression profiles of *MdMADS16* mRNA in floral organ and fruit developmental stages of an apple tree by in situ hybridization.

A: Young flower bud with inflorescence meristem,

B: Young flower bud at the very early stage of differentiation into floral organ having floral primordium,

C: Flower bud in the process of differntiating into floral organ,

D: Immature fruit at 7-10 days after pollination,

c - carpel primordium;

ft - floral tube;

im - inflorescence meristem;

l - leaf appendage;

o - ovule;

st - stamen primordium

Fig. 7 is a photograph showing the result of PCR amplifying *nptⅡ* gene using genomic DNA of a transformant, in order to confirm the insertion of a recombinant *pMdMADS14* plasmid in a transgenic tomatos.

Lane a: Wild type,

Lane b: *MdMADS14 anti-sense,*

Lane c: *MdMADS14 sense 1,*

Lane d: *MdMADS14 sense 2*

27

Fig. 8 is a photograph showing the fruit of T1 generation of *MdMADS14 sense 1* in which ripening is delayed, compared with wild type.

5   Fig. 9 is a set of photographs showing the comparison of germination rate between *sense 1* seeds (T2 generation), shown in Fig. 8, and wild type seeds, which had been cultured for 5 days under the same conditions.

10   Fig. 10 is a set of photographs showing the comparison of germination rate between *sense 1* seeds (T2 generation), shown in Fig. 8, and wild type seeds. Before the comparison, seeds of both groups were collected and stored in cool dry condition for one year, and then they 15   were cultured for 5 days under the same conditions.

Fig. 11 is a set of photographs showing the result of comparison of fruits between *MdMADS14 sense 2* and wild type.

20

Fig. 12 is a set of photographs showing the results of *MdMADS14 anti-sense* phenotype analysis. Fig. 12(a) shows the overall appearances of MdMADS14 *anti-sense* and wild type, Fig. 12(b) is a histological observation of 25   flower and carpel before pollination, and Fig. 12(c) is a

histological observation of early fruit and ovule after pollination.

Fig. 13 is a photograph showing the result of PCR amplifying *nptII* gene using genomic DNA of a transformant, in order to confirm the insertion of a recombinant *pMdMADS16* plasmid in a transgenic tomatos.

Lane a: Wild type,

Lane b: *MdMADS16 anti-sense*,

Lane c: *MdMADS16 sense 1*,

Lane d: *MdMADS16 sense 2*

Fig. 14 is a set of photographs showing that *MdMADS14 sense 1* and *MdMADS16 sense 1* have the common phenotype of early seed germination inside of a fruit, compared with wild type.

Fig. 15 is a set of photographs showing that *MdMADS16 sense 2* and *MdMADS14 sense 2* have the common phenotype that sepal is changed into fruit flesh and pathenocarpic fruit is formed as shown in each cross section of fruit.

Fig. 16 is a set of photographs showing overall appearances of *MdMADS16 anti-sense* and wild type (upper),

and a magnified view thereof (lower) indicating no fruit formation.

Fig. 17 is a set of photographs showing the expression levels of *RIN* gene in the fruits of *MdMADS14 senses* and *MdMADS16 senses*, which were quantified by RT-PCR and PCR-Southern blot.

Lane a: Wild type,

Lane b: Fruit of *MdMADS14 sense 1*,

Lane c: Fruit of *MdMADS16 sense 1*,

Lane d: Fruit of *MdMADS14 sense 2*,

Lane e: Fruit of *MdMADS16 sense 2*

Fig. 18 is a set of photographs showing the expression levels of *Le20ox-1* gene in the fruits of *MdMADS14 senses* and *MdMADS16 senses*, which were quantified by RT-PCR and PCR-Southern blot.

Lane a: Wild type,

Lane b: Fruit of *MdMADS14 sense 1*,

Lane c: fruit of *MdMADS16 sense 1*,

Lane d: Fruit of *MdMADS14 sense 2*,

Lane e: fruit of *MdMADS16 sense 2*

Fig. 19 is a set of photographs showing the expression levels of *Le20ox-1* gene in leaves of *MdMADS14* senses, anti-senses, *MdMADS16* senses and anti-senses, which were quantified by RT-PCR and PCR-Southern blot.

5   Lane a: Wild type,

Lane b: Leaves of *MdMADS14 sense 1,*

Lane c: Leaves of *MdMADS16 sense 1,*

Lane d: Leaves of *MdMADS14 sense 2,*

Lane e: Leaves of *MdMADS16 sense 2,*

10   Lane f: Leaves of *MdMADS14 anti-sense,*

Lane g: Leaves of *MdMADS16 anti-sense*

【Best Mode】

Practical and presently preferred embodiments of the

15   present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope

20   of the present invention.

<u><Example 1> Clonning of an MADS-box gene from apple and nucleotide sequence analysis thereof</u>

cDNA library (SUNG. S.K. *et al., Mol. cells,* 8: 565-577, 1998) constructed from pistil of apple flower (*Malus*

25   *domestica* Borkh cv. Fuji) was used for the preparation of

cDNA pool by *in vivo excision* (Stratagene, USA, Catalog #200450). The nucleotide sequence of MADS-box genes was searched from GenBank (NIH, USA), a database of nucleotide sequences of genes, and then a set of forward and reverse primer was designed from the most conservative region of the nucleotide sequence, which are represented by SEQ. ID. No 3 and SEQ. ID. No 4, respectively. The degenerate PCR was then carried out on pistil cDNA pool, constructed from apple flowers, as a template using the forward and reverse primer set.

The PCR reaction mixture contains 100 ng of cDNA template, 10 µM of each forward and reverse primer, 20 mM of Tris-HCL (pH 8.4), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each of dNTP, and 2.5 unit of *taq* polymerase (*Thermus aquaticus* DNA polymerase) (TaKaRa, Japan). The PCR reaction was carried out on a thermal cycler (Perkin Elmer 9600, Perkin Elmer) under the following cycling condition: initial denaturation at 94°C for 3 min; 1 cycle of denaturation, annealing and extension at 94℃/30 seconds, 60℃/1 minute, and 72℃/1 minute, respectively; followed by a touch down PCR where the annealing temperature is decreased by 1℃ every cycle to 50℃(touchdown annealing temperature) At the last annealing temperature (50℃), 10 cycles were performed, and final extention at 72℃ for 5 minutes to complete the

PCR processes. The resulting PCR product was then diluted
10 fold and used as a template for subsequent nested
degenerated PCR using forward primer set forth in SEQ. ID.
No 5 and reverse primer set forth in SEQ. ID. No.4 The
5   PCR reaction was carried out under the same condition as
above The PCR product was cloned into pGEM-T Easy vector
(Promega, USA) and then nucleotide sequence of the product
was investigated by using T7 primer and SP6 primer.

10   The resulting nucleotide sequence data was then
analyzed by homology analyzing program (blast, NCBI, USA)
and was found to be a partial sequence of the MADS-box
gene. Subsequently, a reverse primer set forth in SEQ. ID.
No 7 based on the nucleotide sequence information of the
15   above, and a forward primer set forth in SEQ. ID. No 6
based on the nucleotide sequence containing restriction
enzyme site of pBluescriptSK(-) vector (Stratagene, USA)
were then designed and used for a subsequent PCR reaction.
The PCR in the same reaction buffer as above was performed
20   under the following cycling condition; initial
denaturation at 94℃ for 3 minutes; 35 cycles of
denaturation, annealing and extension at 94℃/30 seconds,
60℃/1 minute, and 72℃/1 minute, respectively; and final
extenstion at 72℃ for 5 minutes to complete the reaction.
25   A 450 bp PCR product thus obtained was then cloned into a

pGEM-T Easy vector and the nucleotide sequence of the cloned fragment was investigated by using T7 primer and SP6 primer. The nucleotide sequences data thus obtained were analyzed by homology analyzing program (blast, NCBI, USA) and found to correspond to 5' end of MADS-box gene having 687bp. Subsequently, based on the resulting sequence information, a forward primer represented by SEQ. ID. No 8 and reverse primer represented by SEQ. ID. No 9 were designed for a subsequent PCR. The PCR in the same reaction buffer as above was performed under the following cycling condition; initial denaturation at 94℃ for 3 minutes; 35 cycles of denaturation, annealing and extension at 94℃/30 seconds, 60℃/1 minute, and 72℃/1 minute, respectively; and final extenstion at 72℃ for 5 minutes to complete the reaction. An amplified product about 910 bp in size thus obtained was then cloned into a pGEM-T Easy vector. The resulting plasmid was then used for a transformation of XL1-Blue MRF', an *Escherichia coli* strain, and then isolated therefrom.

Nucleotide sequence of the isolated plasmid was investigated. As a result, full-length sequences of two different genes, as shown in Fig. 1 and Fig. 2, were identified which were highly homogenous but had different amino acid sequence.

The full-length sequences thus obtained were each represented by SEQ. ID. No 1 (Fig. 1) and by SEQ. ID. No 2 (Fig. 2). Further, the nucleotide seuence set forth in SEQ. ID. No 2 were then analyzed by clustal method using

5   MegAlign (DNAstar Inc., USA), a program for sequence analysis and was confirmed to be a novel gene having different nucleotide sequence from that represented by SEQ. ID. No 1. As shown in Fig. 1, the gene set forth in SEQ. ID. No.1 contains an open reading frame (ORF) encoding 242

10   amino acids, and also a nucleotide sequence encoding MADS-domain at its *N*-terminal region. Accordingly, it was designated 'MdMADS14'. As shown in Fig. 2, the gene set forth in SEQ. ID. No 2, which was designated 'MdMADS16', also contains an ORF encoding 242 amino acids of the same

15   size as the one in MdMADS14 gene, and also a nucleotide sequence encoding MADS-domain at its *N*-terminal region. The homology between MdMADS14 and MdMADS16 genes in 242 amino acid sequences was 88.4%, the homology between the two genes in MADS-domain region was 98.3%, and the

20   homology between the two genes in the region other than MADS-domain was over 85% (Fig. 3). High homology of MADS-domain regions seems to be common among the members of MADS-box gene family, thus a gene having homology with MdMADS14 and MdMADS16 gene has to be identified based on

25   the homology data from the region other than MADS-domain.

Thus, it appears that *MdMADA14* gene and *MdMADS16* gene are members of the same family. Furthe it was found that, as shown by the phylogenetic tree of MADS-box genes (Fig. 4),
5   these two genes are more closely related to each other than to any other gene of MADS-box gene group.

<Example 2> Analysis of Expression of *MdMADS14* and *MdMADS16 genes* in various organs and in each different developmental stage using real-time PCR

Real-time PCR was performed to investigate the
10   expressions of *MdMADS14* gene and *MdMADS16* gene in a variety of organs and in various developmental stages of fuits. Test samples were taken from various parts of an apple tree, i.e., leaves, flower buds, flowers, young fruit at 2 weeks after blomming and at 24 weeks after
15   blomming. Total RNA was extracted from each of the above samples and used for real-time PCR was performed with it.

The expression levels of the genes in each organ and in developmental stage were quantified by real-time PCR using SYBR Green Master Mix Kit (Applied Biosystems, USA)
20   as recommended by the supplier. Specifically, the first strand cDNA pool was constructed from 2 μg of total RNA extracted from each of the samples above. Primer concentrations were first optimized such that non-specific PCR products or a primer dimmer was not formed during the
25   PCR. The optimum concentration determined for each primer

was as follows: for *MdMADS14* gene amplification, forward primer of SEQ. ID. No 10 and reverse primer of SEQ. ID. No 11 were 50 nM and 300 nM, respectively; for *MdMADS16* amplification, forward primer of SEQ. ID. No 12 and

5   reverse primer of SEQ. ID. No 13 were both 900nM; and for actin gene amplification as an internal control, forward primer of SEQ. ID. No 14 and reverse primer of SEQ. ID. No 15 were both 900 nM.

Each primer set was designed such that the size of

10 amplified products is not more than 120 bps for accurate measurement within a short reaction time possible. Real-time PCR was performed on the first strand cDNA pool from each organ and developmental stage using a primer set specific for each gene to be detected at optimum

15 concentrations as described above. The PCR was carried out on a thermal cycler for real-time PCR (7300 Real-time PCR system, Applied Biosystems, USA) under the following cycling condition: initial incubation at 50℃ for 2 minutes to activate polymerase therein; followed by

20 denaturation at 95℃ for 10 minutes; 40 cycles of denaturation at 95℃ for 15 seconds and hybridization or annealing at 60℃ for 1 minute. The signal intensity from fluorescent dye in each amplified product was detected in a hybridization or annealing step of every cycle of RT-PCR

25 to quantify the PCR product amplified. The expressions

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levels of *MdMADS14* gene and *MdMADS16* gene in each organ and developmental stage were then determined and normalized against the amount of actin synthesized.

As shown in Fig. 5, the expression of *MdMADS14* gene was observed from flower buds to the young fruit development stage, while it is rarely expressed in leaves and mature fruits of an apple tree. The expression pattern of *MdMADS16* gene was similar to the one shown by *MdMADS14* gene, only the expression levels of *MdMADS16* gene were lower that of *MdMADS14* gene. In order to examine specificity of each reaction product, melting curve analysis was performed. As a result, *MdMADS14* and *MdMADS16* were confirmed to be different from each other, showing different melting temperature.

<Example 3> mRNA Expression of *MdMADS16 gene* by *in situ* hybridization

The expression of mRNA of *MdMADS16* gene in young flower bud and flowers after pollination from an apple tree was analyzed by *in situ* hybridization. The sample cut at appropriate size was fixed in a fixing solution, and embedded in paraffin block. The paraffin block was then sectioned at 10 μm thick followed by hybridization with a approproate nucleotide probe. The probe used

herein was directed to a region other than MADS-domain and K-domain that are commonly found in MADS-box genes, and was prepared by labelig the antisense strand of *MdMADS16* gene with DIG. The probe represented by SEQ. ID. No 16 was

5   preferably used in this invention. However, the difference between the two genes was not distinguished by the probe, because homology in those regions between *MdMADS16* gene and *MdMADS14* gene was more than 88% (88.1%). In conclusion, as shown in Fig. 6, *MdMADS16* gene was strongly expressed

10   in inflorescence meristem and in ovule. The result indicates that *MdMADS16* gene is involved in fruit and seed development and flower organ formation.

<u><Example 4> Generation of a transgenic plant conatining *MdMADS14* gene</u>

15   DNA isolated from apple containing *MdMADS14* gene as in Example 1 was digested with *Pst* I and *Hind*Ⅲ, then the resulting frament was cloned into a pBluescriptSK(-) vector (Stratagene, USA) digested with the same enzymes.

A recombinant vector for over-expression was

20   constructed by digesting it with *Xba* I and *Cla* I and then cloning forwardly into pGA1530 vector, a vector for plant transformation, pre-digested with the same enzymes. A recombinant vector for the inhibition of gene expression was also constructed by digesting recombinant

25   pBluescriptSK(-) vector containing *MdMADS14* gene with

Xba I and *Hind* Ⅲ, and cloning it reversely into pGA1530 vector, a vector for plant transformation, pre-digested with the same enzymes. The pGA1530 vector for plant transformation contains 35S promoter, T7 terminator and npt Ⅱ (*neomycin phosphotransferase*) as a selection marker which provides kanamycin resistance (*Plant molecular Biology Manual*, 1988, A3:1-19). The resulting recombinant plasmid containing *MdMADS14* gene in forward and reverse direction was designated 'pMdMADS14', and 'pMdMADS14-R', respectively. The recombinant plasmids were then used to tranform *Agrobacterium tumefaciens* LBA4404 (A. Hoekema *et al.*, 1983, *Nature*, 303, 179-181), and each transformed *Agrobacterium tumefaciens* containing 'pMdMADS14' or 'pMdMADS14-R' was selected in a growth medium containing Kanamycin, which were deposited with Korean Collection for Type Cultures (KCTC) of Korea Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession No: KCTC 10588BP).

Minitomato (*Lycopersicon esculentum* cv. 'Micro-Tom') was used for the generation of a transgenic plant into which pMdMADS14 was transferred. In order to select a target tissue, mature seeds of minitomato were first sterilized by immersion in 70% ethanol for 1 minute, washing with sterilized water three times, immersion in 2% NaOCl (Sodium hypochloride) for 15 minutes, followed by

washing with sterilized water more than 7 times.   The
seeds were then transferred to a plate containing seed
germination medium (1/2 MS, 3% sucrose, 0.8% agar). 14
days later, seeds began to be germinated. As cotyledon was
5   opened and true leaf began to be sprout, the cotyledon was
selected as a target tissue for transformation.

The recombinant *Agrobacterium tumefaciens* LBA4404,
prepared as in the above Example, was cultured in YEP
10   medium (1% yeast extract, 1% peptone, 0.5% NaCl)
containing 50 mg/ℓ of kanamycin until OD_{600} was 0.8.   The
cultured Agrobacterium was co-cultured in 1/2 MS medium
supplemented with 200 μM of acetosyringone with shaking at
150 rpm, at 22℃ for 2 hours.   Then, the target tissue,
15   cotyledon section of a minitomato prepared as above, was
added thereto and co-cultured with shaking at 150 rpm, at
22℃ for 10 minutes.   The resulting cotyledon sections
were then placed onto a plate containing co-culture medium
(MS, IAA 1 μM, zeatin 10 μM, sucrose 3%, acetosyringone
20   200 μM, agar 0.7%) and cultured for 2 days.   Then, the
cotyledon sections were transferred onto a regeneration
medium (MS, IAA 1 μM, zeatin 10 μM, sucrose 3%, cefotaxime
350 mg/ℓ, kanamycin 50 mg/ℓ, agar 0.7%), followed by two
sub-cultures in succession at three weeks interval. Then,
25   shoots generated from the cotyledon sections were

transferred onto selective root-inducing medium (MS 1/2, IAA 1 µM, sucrose 3%, kanamycin 50 mg/ℓ, agar 0.7%) containing kanamycin to select transformants. The transformed shoots can be differentiated from untransformant since the untransformant undergoes necrosis accompanied by color change while transformed shoots were growing normally with roots. Shoots with roots were then transferred onto soil after acclimatization.

As a result, transformants over-expressing *MdMADS14* gene (senses) showed two phenotypes, each of which was designated "transformant 1" (*MdMADS14 sense 1*) and transformant 2 (*MdMADS14 sense 2*). Another transformant in which the expression of *MdMADS14* gene was suppressed (*anti-sense*) showed the phenotype of no-fruit formation, which was designated "inhibited transformant 1" (*MdMADS14 anti-sense*). As shown in Fig. 7, the successful integration of *MdMADS14* gene into the genome of each of the above transformant was verified by PCR amplifying *nptII* gene in each transformant. A set of forward and reverse Primers used for the PCR to confirm the integration of *nptII* gene is represented by SEQ. ID. No 17 and by SEQ. ID. No 18, respectively.

<Example 5> Analysis of the functions of *MdMADS14* gene in a transgenic plant

Phenotypes of *MdMADS14* *senses* and *anti-sense*, prepared in the above example 4, were investigated to analyze the functions of *MdMADS14* gene in plants.

As a result, *MdMADS14* *senses* showed two different

5 phenotypes; *sense 1* showed delayed ripening but promoted seed germination and *sense 2* showed the characteristics of that sepal was changed into fruit flesh and parthenocarpic fruit was formed.

As shown in Fig. 8 and Fig. 9, *sense 1* showed

10 delayed fruit ripening, comparing to wild type, but promoted seed germination. Such characteristics were observed not only in T1 generation but also in T2 generation, indicating that the characteristics are inherited.

15 In order to confirm the above result, germination speed and germination rate of seed (T2 generation) of *sense 1* were measured (Table 1, measurement of the primary germination rate). Specifically, 25 seeds each from wild type tomato and *MdMADS14* *sense 1* as shown in Fig. 8 were

20 harvested, and then dried for 10 days. Subsequently, the seeds were placed on a filter paper moisten with distilled water in a petridish and cultured at 25℃ for 5 days. Germination speed for each group was measured and statistically analyzed. As shown in Fig. 9, seed

25 germination in *sense 1* was much promoted than in wild type.

As shown in Table 1, while the average germination speed of seeds in *sense 1* was 1.77 day, the average germination speed of seeds in wild type was 3.8 days. Moreover, germination rate of seed in *sense 1* was twice as high as the wild type. Wild type seeds and seeds (T2 generation) of *sense 1* were stored in cool/dry condition for a year, then germination speed and germination rate were investigated again by the same method as used above (Table 2, measurement of the second germination rate). As a result, as shown in Fig. 10, seed germinaton speed and gerimination rate in *sense 1* was still promoted, comparing to wild type. As shown in Table 2, the average germination speed of *sense 1* seeds was 3 days, while the average germination speed of wild type tomato seeds was 6 days, indicating that germination speed was shortened in *sense 1* twice as much as in wild type, and germination rate in *sense 1* was still two-fold higher than in wild type. After one-year storage, germination speed and germination rate were slow and dull in general, but the speed and rate in *sense 1* were still about two-fold higher than those in wild type.

【Table 1】

	Seeds from Transformant 1 (T2)	Seeds from non-transformant

Average Germination Term	1.77 Days	3.8 Days
Germination Rate	100%	50%

【Table 2】

	Seeds from Transformant 1 (T2) ; 1 year storage in a cool/dry place.	Seeds from Non-transformant ; 1 year storage in a cool/dry area
Average Germination Term	3Days	6Days
Germination Rate	92%	40%

MdMADS14 sense 2 showed such phenotype as sepal was changed into fruit flesh and parthenocarpic fruit was formed. As shown in Fig. 11, sepal in *sense 2* was changed into fruit flesh with being corpulent and parthenocarpic fruit was formed without seed formation, unlike wild type.

In the meantime, *MdMADS14 anti-sense* plant showed common phenotype that was characterized by no-fruit formation. As shown in Fig. 12, the development of floral organ in the early stage was not affected (Fig. 12(a) and Fig. 12(b)) but seed development after pollination was inhibited, and so fruits were not grown enough (Fig. 12(c)). Floral organ and ovary of each developmental stage in *anti-sense* were fixed in a fixing solution (50mM PIPES; ph 6.8, 4% paraformaldehyde), leading to plastic

embedding. The samples were cut by 7 μm and stained with toluidine blue O, followed by histological observation.

As a result, as shown in Fig. 12(b) and 12(c), the development of ovule in early developmental stage of floral organ was not much affected, but the development of ovule and ovary after pollination was clearly inhibited.

<Example 6> Generation of a transgenic plant having MdMADS16 gene and analysis of the functions of MdMADS16 gene in the plant

DNA isolated from apple containing MdMADS16 gene as in Example 1 was digested with PstI and HindIII and the resulting fragment was cloned into a pBluesecriptSK(-) vector (Stratagene, USA) digested with the same enzymes. The vector was digested with BamHI and HindIII again, followed by fill-in with the fragment containing the above gene. Then, MdMADS16 gene was sub-cloned into pRTL2 vector (Restrepo, M. et al., Plant Cell, 1990, 2: 987-998) pre-digested with SmaI. The plasmid prepared by the above procedure included both forward cloning and reverse cloning of MdMADS16 gene, so both forwardly cloned MdMADS16 gene and reversely cloned MdMADS16 gene were separated through nucleotide sequencing. Each of them was digested with HindIII, followed by cloning into pCAMBIA 2301 vector (CAMBIA, Australia) for plant transformation,

which was pre-digested with the same enzyme. pCAMBIA 2301 vector for plant transformation contains 35S promoter, *nos* (*nopaline synthase*) and *npt II* (*neomycin phosphotransferase*) as a marker gene, which provides

5 kanamycin resistance. The recombinant plasmid in which *MdMADS 16* was forwardly inserted was designated 'pMdMADS16' and the recombinant plasmid in which *MdMADS16* gene was reversely inserted was designated 'pMdMADS16-R'. Those recombinant plasmids were inserted in *Agrobacterium*

10 *tumefaciens* LBA4404, and then strains containing those recombinant plasmids, pMdMADS16 and pMdMADS16-R, were selected from kanamycin selection medium. The selected *Agrobacterium tumefaciens* containing the recombinant plasmid pMdMADS16 was deposited at Korean Collection for

15 Type Cultures (KCTC) of Korea Institute of Bioscience and Biotechnology (KRIBB) on January 30, 2004 (Accession No: KCTC 10589BP), which was further used for Agrobacterium tumefaciens-mediated transformation of minitomato plant as described in Example 3. *MdMADS16* senses, transgenic

20 minitomato plant generated by the above procedure, showed two phenotypes and was designated *MdMADS16 sense 1* and *MdMADS16 sense 2* depending on their phenotypes. The phenotypes were the same as those of *MdMADS14 senses*. In other words, *MdMADS16 sense 1*, as shown in Fig. 14, showed

25 delayed ripening but promoted seed germination like

MdMADS14 sense 1. MdMADS16 sense 2, as shown in Fig. 15, showed the phenotype of transformation of sepal into fruit flesh as shown in Fig. 16 like *MdMADS14 sense 2. MdMADS16 anti-sense* showed phenotype of no-fruit formation after pollination, like *MdMADS14 anti-sense,* which indicate that both *MdMADS14* gene and *MdMADS16* genes are involved in furit formation.

From the Examples above, it can be known that *MdMADS14* gene and *MdMADS16* gene belong to the same family, showing high homology, and are involved in seed and fruit development from the functional analysis of transgenic plants. Such functions of *MdMADS14* and *MdMADS16* gene of the present invention are novel, which has not been found previously in currently known MADS-box genes.

<Example 7> Analysis of the expressions of *RIN* gene in *MdMADS14* and *MdMADS16* senses by RT-PCR

Both *MdMADS14 sense 1* and *MdMADS16 sense 1* generated in Example 4 and Example 6, respectively, show the same phenotype as was shown for the tomato plant in which the ethylene synthesis is inhibited during ripening (Julia Vrebalov *et al.,* SCIENCE, 2002, 296: 343-346). Thus, *RIN* gene expression that plays an important role in ethylene synthesis during ripening of tomato was investigated in

MdMADS14 sense 1 and *MdMADS16 sense 1* by reverse transcription-polymerase chain reaction (RT-PCR).

Specifically, the first strand cDNA pool was prepared from each 2 μg of total RNA extracted from fruits of *senses* and wild type using reverse transcriptase primed with oligo(dT) primer. RT-PCR was then performed by using the cDNA pool as a template according to the method known in the pertinent art. Particulary, the PCR conditions were set to assure the validity of the analysis such that the amplified products are in exponential phase. Actin primer, used in the above example 2, was used for internal control.

In order to confirm whether or not an introduced gene was expressed in a transformant, forward primer represented by SEQ. ID. No 19 and reverse primer represented by SEQ. ID. No 20 were prepared based on common nucleotide sequence region (referred as MdMADS) between *MdMADS14* gene and *MdMADS16* gene, and then used for the amplification of MdMADS. Forward primer represented by SEQ. ID. No 21 and reverse primer represented by SEQ. ID. No 22 were used for the amplification of *RIN* gene. PCR was performed under the following cycling condition: initial denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute and extention at 72°C for 1 minute; and final extension at 72°C for 10 minutes. The obtained PCR

products were then electrophoresed on an agarose gel, and the gel was transferred onto a Hybond N+ membrane according to a method known in the pertinent art. The membrabe was then allowed to hybridize with the probe for

5   *RIN* gene, *MdMADS* gene and actin gene, each of which was labeled with biotin, and the signals from each probe hybridized to its corresponding target sequence were detected using Southern-Star™ System (Applied biosystems, USA) recommended by the supplier.

10   The results are shown in Fig. 17. First, the successful expression of the gene introduced into each transformatn was confirmed by the signals detected only in transformants but not in a non-transformant. Further, the expression of *RIN* gene was reduced in *MdMADS14 sense 1* and

15 *MdMADS16 sense 1*, which is consistent with delayed ripening. On the contrary, the expressions of *RIN* gene in *MdMADS14 sense 2* and *MdMADS16 sense 2* that forms parthenocarpic fruit were not much different from that of wild type.

20   It has been known that reduced expression of *RIN* gene represents the reduced ethylene synthesis during ripening since *RIN* gene plays an essential role in ethylene sythesis during ripening (R.C. Herner *et al.*, *Plant Physiol.*, 1973, 52: 38-42; Julia Vrebalov *et al.*,

25 *SCIENCE*, 2002, 296: 343-346). Therefore, the results from

this experiment indicate that the delayed ripening in both *MdMADS14 sense 1* and *MdMADS16 sense 1* was resulted from the decrease of ethylene synthesis by the introduction of *MdMADS14* and *MdMADS16* gene. Further, it indicates that *MdMADS14* gene and *MdMADS16* gene of the present invention can be effectively used for the regulation of fruit development and fruit ripening delay.

<Example 8> Analysis of the expression of *Le20ox-1* gene in a transformant harboring *MdMADS14* gene or *MdMADS16* gene by RT-PCR

    In order to examine the level of active gibberellin synthesized in *MdMADS14 senses* and *MdMADS16 senses* obtained in Example 4 and Example 6, the expression level of *Le20ox-1* gene was investigated. As mentioned hereinbefore, the mRNA synthesis of *Le20ox-1* gene, which is involved in the conversion of C-20 into C-19 gibberellin followd by activation by 3β-hydroxylase leading to active gibberellin, is regulated by negative feedback from high level of active gibberellin present in a cell. Thus, the mRNA level of *Le20ox-1* gene is negatively correalated with the amount of active gibberellin present.

    The expression levels of *Le20ox-1* gene in fruits and leaves from transgenic plants and wild type were compared

and determined by RT-PCR followed by Southern blot as described in Example 7 except that, for the amplification of *Le20ox-1* gene, forward primer represented by SEQ. ID. No 23 and reverse primer represented by SEQ. ID. No 24 were used.

The results are shown in Fig. 18. The expression of *Le20ox-1* gene in fruits of *MdMADS14 senses* and *MdMADS16 senses* were significantly decreased, compared with that of wild type, which suggests that synthesis of active gibberellin in *MdMADS14 senses* and *MdMADS16 senses* was much increased, comparing to that in wild type.

The expression levels of *Le20ox-1* gene in leaves of *MdMADS14 senses*, *MdMADS16 senses*, *MdMADS14 anti-sense* and *MdMADS16 anti-sense* were also compared. The results are shown in Fig. 19. The expression of *Le20ox-1* gene were significantly decreased in leaves of *MdMADS14 senses* and *MdMADS16 senses*, compared wtih wild type, but the expression of *Le20ox-1* gene were increased in leaves from *MdMADS14 anti-sense* and *MdMADS16 anti-sense*. The results indicate that synthesis of active gibberellin in *MdMADS14 senses* and *MdMADS16 senses* was much increased, but synthesis of active gibberellin in *MdMADS14 anti-sense* and *MdMADS16 anti-sense* was much decreased, comparing to that in wild type.

Therefore, *MdMADS14* gene and *MdMADS16* gene of the present invention can be effectively used for the regulation of active gibberellin synthesis.

5   【Industrial Applicability】

The genes of the present invention regulate fruit and seed development by inhibiting or promoting active gibberellin synthesis. A transgenic plant containg the gene of the present invention exhibits phenotype that is

10   characterized by promoted seed developmemt, transformation of calyx into fruit flesh and formation of parthenocarpic fruit. Thus, the genes of the present invention can be effectively used for the regulation of active gibberellin synthesis, production of parthenocarpic fruit, and fruit

15   and seed development, all of which are important in incrasing the productivity of crops.

【Sequence List Text】

The SEQ. ID. No 1 is a nucleotide sequence of

20   *MdMADS14*,

The SEQ. ID. No 2 is a nucleotide sequence of *MdMADS16*,

The SEQ. ID. No 3, No 4 and No 5 are nucleotide sequences of degenerate primers used for degenerate PCR

25   described in Example 1,

The SEQ. ID. No 6 and No 7 are nucleotide sequences of primers used for PCR to amplify the nucleotide sequence of SEQ. ID. No 1 in Example 1,

The SEQ. ID. No 8 and No 9 are nucleotide sequences

5   of primers used for PCR to amplify the nucleotide sequence of SEQ. ID. No 1 in Example 2,

The SEQ. ID. No 10 and No 11 are nucleotide sequences of primers used for the amplification of *MdMADS14* gene by real-time PCR and RT-PCR described in

10 Example 2,

The SEQ. ID. No 12 and No 13 are nucleotide sequences of primers used for the amplification of *MdMADS16* gene by real-time PCR and RT-PCR described in Example 2,

15 The SEQ. ID. No 14 and No 15 are nucleotide sequences of primers used for the amplification of *actin* gene by real-time PCR and RT-PCR described in Example 2,

The SEQ. ID. No 16 is a nucleotide sequence of probe used in *in situ hybridization* described in Example 3,

20 The SEQ. ID. No 17 and No 18 are nucleotide sequences of primers used for PCR to confirm the expression of *nptII* gene described in Example 4,

The SEQ. ID. No 19 and No 20 are nucleotide sequences of primers used for real-time PCR and RT-PCR for

25 the amplification of *MdMADS* gene descrined in Example 7,

The SEQ. ID. No 21 and No 22 are nucleotide sequences of primers used for real-time PCR and RT-PCR for the amplification of *RIN* gene described in Example 7,

The SEQ. ID. No 23 and No 24 are nucleotide sequences of primers used for real-time PCR and RT-PCR for the amplification of *Le20ox-1* gene described in Example 8.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes as the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.